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Comprehensive analysis of antioxidant mechanisms in *Arabidopsis* glutathione peroxidase-like mutants under salt- and osmotic stress reveals organ-specific significance of the AtGPXL's activities

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ABSTRACT

Plant glutathione peroxidases contain cysteine in their active site instead of selenocysteine, and most of them use the thioredoxin (TRX) system more efficiently than the glutathione (GSH) system during the reduction of H_2O_2 and lipid peroxides. Recently, the more precise glutathione peroxidase-like (GPXL) name was adopted for the eight *Arabidopsis thaliana* isoenzymes. In this paper we have compared the effect of osmotic and salt stresses on the 6-week-old T-DNA insertion mutants (*Atgpxl1-8*) grown hydroponically. The glutathione peroxidase (GPOX) activity measured with cumene hydroperoxide substrate in the wild type *Arabidopsis* shoots and roots was 2–5 times higher than the thioredoxin peroxidase (TPOX) activity. Mutation in one of the eight *AtGPXLs* resulted in decreased TPOX activity in untreated shoots and, in contrary to the wild type, this activity did not increase under stress, verifying the connection with TRX system. The level of reduced ascorbate significantly altered in shoots and the amount of GSH in roots under both control conditions and after 2 days of stress treatments. While positive correlations were found between GSH and TPOX activity in the wild type shoots and roots, the connection between the AtGPXLs and the GSH pool was stronger in roots than in shoots. Nevertheless, the TPOX activity increased in *Atgpxls* roots when the GSH content decreased, indicating the relationship between the GSH and TRX systems. The *AtGPXL* expression in mutant plants showed that some isoenzymes are regulated jointly. Furthermore, while *AtGPXLs* were generally down-regulated, several stress-inducible transcription factor genes were up-regulated, especially after applying osmotic stress.

1. Introduction

In plant cells, disadvantageous environmental conditions increase the production of reactive oxygen species (ROS). The ROS, such as superoxide radical, hydroxyl radical or hydrogen peroxide (H_2O_2), can induce detrimental oxidation of macromolecules including lipids, proteins and nucleic acids. In order to minimize ROS-derived damages and to keep its levels tightly regulated, a series of non-enzymatic antioxidants including but not limited to ascorbate (ASC), glutathione (GSH), carotenoids and tocopherols, as well as a set of enzymatic ROS detoxification systems have evolved in aerobic organisms which are represented in various combinations in each intracellular organelle (Noctor et al., 2014). Among the enzymes, the catalase (CAT) and different peroxidases may be implied in mass scavenging of H_2O_2 (Asada, 1992; Willekens et al., 1995). Guaiacol peroxidases (POXs) catalyse the reduction of H_2O_2 using electrons from various donor molecules (Passardi et al., 2004), the ascorbate peroxidase (APX) reduces H_2O_2 at the expense of ASC (Asada, 1988), while glutathione peroxidases (GPXs), glutathione transferases (GSTs), and peroxiredoxins (PRXs) reduce H_2O_2 and hydroperoxides by thiol-mediated pathways (Dietz et al., 2002; Chang et al., 2009). The role and mechanism of most of these enzymes in stress responses have been investigated intensively for several decades, even though relatively little is known about plant GPXs. While the evidence of their activity in different cultured plant cells was first reported already in 1985 (Drotar et al., 1985), several plant GST isoenzymes also have glutathione-

Abbreviations: ASC, ascorbate; APX, ascorbate peroxidase; CAT, catalase; CDNB, 1 chloro-2,4-dinitrobenzene; CHP, cumene hydroperoxide; DHA, dehydroascorbate; GPOX, glutathione peroxidase; GPXL, glutathione peroxidase-like; GR, glutathione reductase; GSH, reduced glutathione; GSSG, glutathione disulfide, oxidized glutathione; GST, glutathione transferase; MDA, malondialdehyde; POX, guaiacol peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; TPOX, thioredoxin peroxidase; TRX, thioredoxin

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dependent peroxidase activity (Roxas et al., 1997; Kilili et al., 2004; Basantani and Srivastava, 2007; Dixon and Edwards, 2009; Dixon et al., 2009), encumbering to clarify the significance of the particular GPX or GST enzymes.

The GPX (EC1.11.1.9) enzyme family comprises proteins phylogenetically related to non-heme thiol peroxidases that catalyse the reduction of H_2O_2 or organic hydroperoxides to water or the respective alcohols using reduced glutathione. Characterisation of numerous GPXs in various organisms revealed their broad substrate specificities and high affinity to H_2O_2 (Brigelius-Flohé and Flohe, 2003). Their ROS-scavenging role was proved in several reports (Arthur, 2001; Battin and Brumaghim, 2009; Yang et al., 2015). The mammalian GPXs are central components of the antioxidant metabolism and participate largely in the repair of biomembranes (McCay et al., 1976; Imai and Nakagawa, 2003; Islam et al., 2015). The plant glutathione peroxidase genes are closely related to animal phospholipid hydroperoxide glutathione peroxidases (Margis et al., 2008), however the isoenzymes contain cysteine instead of selenocysteine in their active site contrary to most of the animal GPXs (Herbette et al., 2007). While some of them have both glutathione peroxidase and thioredoxin peroxidase (TPX) functions, they prefer the thioredoxin (TRX) regenerating system *in vitro* rather than the glutathione system and even are regarded to be actually thioredoxin peroxidases (Herbette et al., 2002; Iqbal et al., 2006; Navrot et al., 2006; Lubos et al., 2011). They reduce more efficiently peroxides different from H_2O_2 such as organic hydroperoxides and lipid peroxides (Milla et al., 2003). Recently, Attacha et al. (2017) adopted the GPX-like (GPXL) nomenclature for the *Arabidopsis thaliana* isoforms to avoid any confusion resulting from protein names (http://www.arabidopsis.org/servlets/TairObject?type=gene_class_symbol&id=6531241478).

Passaia et al. (2014b) investigated the role of *Arabidopsis thaliana* GPXLs in shoot and root development using T-DNA insertion mutant lines. They found, that the shoot phenotypes were largely similar in *Atgpxl* mutants and wild type, however all mutants showed altered root phenotypes. They have confirmed the connections between the AtGPXLs and auxin, abscisic acid, strigolactone hormones, thereby demonstrating the importance of AtGPXLs in the hormone-mediated regulation of lateral root development. Earlier it was reported that redox processes involving glutathione and thioredoxins exert a strong influence on root architecture (Benitez-Alfonso et al., 2009; Bashandy et al., 2010), moreover GSH is involved in the interplay between auxin and strigolactone signaling that controls this process (Marquez-Garcia et al., 2014). It was suggested that GPXLs may be required to GSH- and reduced thioredoxin-mediated redox control of lateral root development (Passaia et al., 2014b).

The key redox pairs in the soluble phase of the cells are the NAD(P)H/NAD(P)$^+$, ascorbate/dehydroascorbate (ASC/DHA), glutathione/glutathione disulfide (GSH/GSSG), and reduced thioredoxin/oxidized thioredoxin (TRX_{red}/TRX_{ox}) – all of which are linked to the production or enhanced availability of ROS. There is a close interplay among the individual redox active molecules, and the status of each of them can influence the plant metabolism and environmental responses. The appropriate cellular response requires the presence of redox-sensitive proteins that can undergo reversible oxidation/reduction and may switch 'on' and 'off' depending upon the cellular redox state (Potters et al., 2010). Redox-sensitive metabolic enzymes may directly modulate corresponding cellular metabolism, whereas redox-sensitive signaling proteins execute their function via downstream signaling components, such as kinases, phosphatases, and transcription factors. A tight control is necessary to balance these activities and maintain coordination, including reversible redox regulation of proteins by dithiol-disulfide exchange, regulation of phosphoproteins, activation of signaling pathways by ROS-responsive regulatory genes (Mittler et al., 2004; Foyer and Noctor, 2005, 2012, 2013). According to present conception, ROS-producing enzymes, antioxidants and their redox states all contribute to the general redox homeostasis in the plant cell (Potters et al., 2010), but glutathione has been considered the master regulator of intracellular redox homeostasis (Foyer and Noctor, 2012; Noctor et al., 2012; Foyer and Noctor, 2013; Gill et al., 2013).

GSH (γ-Glu-Cys-Gly) is an abundant multifunctional tripeptide, which cooperates tightly with ASC in the Foyer–Halliwell–Asada cycle (Foyer and Noctor, 2012, 2013). Besides participating in the reduction of DHA, GSH also plays a key role in the direct ROS scavenging and in the protection of the thiol groups of proteins (Zagorchev et al., 2013). When GSH reacts with oxidants it becomes converted into glutathione disulfide. As a result of the reversible convertibility between the reduced and oxidized forms and the relatively high concentration of the GSH in the cells, glutathione is one of the most important redox buffer systems. It is indicated that beside the amount of the total glutathione, the ratio of the GSH and the GSSG may be an effective marker of cellular redox homeostasis. From the concentrations of reduced and oxidized glutathione the half-cell reduction potential ($E_{GSSG/2GSH}$) can be calculated (Schafer and Buettner, 2001). It was indicated that redox changes (e.g. alteration in H_2O_2 level, ascorbate and GSH concentrations or the ratio of their reduced/oxidized form), and the half-cell reduction potential of the GSH/GSSG couple were correlated with the level of stress tolerance (Soltész et al., 2011).

The involvement of several plant GPXLs in stress responses was already indicated (Bela et al., 2017). Analysis of their gene expression showed that GPXL mRNA steady-state levels usually increase under different biotic and abiotic stresses (Milla et al., 2003; Navrot et al., 2006; Diao et al., 2014; Gao et al., 2014). More detailed investigations revealed that defects of *AtGPXL3* reduced the drought stress tolerance: mutants displayed impaired stomatal closure, faster water loss and lower temperatures of leaves (Miao et al., 2006). Knock out mutation of *AtGPXL8* led to increased sensitivity to salt and osmotic stress compared to wild type (Gaber, 2011), and paraquat treatment suppressed the root growth more and increased the level of oxidized proteins in *Atgpxl8* plants (Gaber et al., 2012). The *Oryza sativa* OsGPXL1 mitochondrial enzyme was proved to be important for root growth, water use efficiency, both phases of photosynthesis and photorespiration under salinity (Lima-Melo et al., 2016). Also, depletion of OsGPXL5 negatively affected the tolerance of salt stress (Wang et al., 2017). Overexpression of wheat *GPXL* genes in Arabidopsis enhanced early tolerance to high salt stress; the transgenic plants showed higher germination rate and decreased growth inhibition by NaCl treatment (Zhai et al., 2013). It was proved that AtGPXL3 does not only have a scavenging function, but can also interact with 2C-type protein phosphatase abscisic acid (ABA) INSENSITIVE2, therefore it acts as an oxidative signal transducer in ABA and drought stress signaling (Miao et al., 2006). Characterisation of the Arabidopsis *root meristemless1 (rml1)* mutant, which has a defect in GSH biosynthesis, revealed altered expression of thioredoxin related genes, among others the *GPXL6*. Presumably that in case of severe glutathione deficiency GPXLs are inclined to use TRX as electron donor compared to GSH, in this way they may have a possible link function between the GSH- and TRX systems (Schnaubelt et al., 2015).

In this paper we have compared the effect of osmotic and salt stresses on hydroponically grown Arabidopsis *gpxl* mutants to investigate roles of individual isoenzymes in the ROS elimination and antioxidant responses. Our aim was to evaluate the connection between the redox states of the main antioxidant redox pairs (ASC and GSH) and the related antioxidant enzyme activities in shoot and root of the different *Atgpxl* mutants under control and stress conditions. Besides investigating the changes of the redox potentials we detected the transcription of *AtGPXLs* and several abiotic stress-related transcription factors (TFs) to reveal their relationship in stress response and in the maintenance of cell homeostasis.

2. Materials and methods

2.1. Plant material

Arabidopsis thaliana (L.) Heynh. (ecotype Columbia) wild type (WT) and T-DNA insertion mutants of the eight glutathione peroxidase (*AtGPXL*) genes (*Atgpxl1-8* mutants) were used. The T-DNA insertion mutant lines were obtained from the Nottingham Arabidopsis Stock Centre (NASC) (*Atgpxl1 [AT2G25080]*: SALK_128885C; *Atgpxl2 [AT2G31570]*: SALK_082445C; *Atgpxl3 [AT2G43350]*: SALK_071176C; *Atgpxl4 [AT2G48150]*: SAIL_623_F09; *Atgpxl5 [AT3G63080]*: SALK_076628C; *Atgpxl6 [AT4G11600]*: WiscDsLox321H10; *Atgpxl7 [AT4G31870]*: SALK_072007C; *Atgpxl8 [AT1G63460]*: SALK_127691C), and the seeds of homozygous genotypes were used. The positions of T-DNA insertions and gene-specific PCR primers used for testing the segregation of T-DNA insertions are shown in Supplemental material (Fig. S1).

2.2. Growth conditions

Plants were grown in Hoagland nutrient solution in a growth chamber (Fitoclima S 600 PLH, Aralab, Rio de Mouro, Portugal) at 21 °C under 100 mmol m^{-2} s^{-1}photon flux density with a 10 h day and 14 h night photoperiod; the relative humidity was 70%. After having grown under standard growth conditions for 6 weeks, some of the plants were treated with 100 mM NaCl or 200 mOsm polyethylene glycol (PEG 6000; −0.489 MPa) (Michel and Kaufmann, 1973) in Hoagland solutions for 2 days, then samples were taken from fully expanded leaves and roots. The experiments were repeated at least three times and the measurements were performed with three replicates unless indicated otherwise.

2.3. Determination of the H_2O_2 level

The H_2O_2 level was measured spectrophotometrically after homogenization of 200 mg shoot or root tissue on ice with 750 µL 0.1% trichloroacetic acid (TCA) as described earlier in Takács et al. (2016). The absorbance of the samples was measured at 390 nm (Uvikon 930 spectrophotometer, Kontron AG, Eching, Germany; which was used for every absorption measurement in our experiments). The amount of H_2O_2 was calculated using a standard curve prepared with 0.1–5 mM H_2O_2 concentrations.

2.4. Determination of malondialdehyde content

Malondialdehyde (MDA) formation was measured with a thiobarbituric acid (TBA)-reactive substances assay, based on the formation of TBA-MDA conjugate (Heath and Packer, 1968). 50 mg shoot or root tissues were homogenized on ice with 0.5 mL 0.1% TCA and 50 µL of 4% butylhydroxytoluene was added to avoid further lipid peroxidation (Benyó et al., 2016). MDA concentrations were calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.5. Measurements of non-enzymatic antioxidants

Ascorbate and glutathione contents were determined according to Horváth et al. (2015a). 300 mg of shoot or root tissue was homogenized with 1.2 mL of 5% TCA. The homogenate was centrifuged at 12 000 *g* for 20 min at 4 °C and the supernatant was used for further determinations.

The ascorbate (ASC) contents were measured spectrophotometrically as was published originally (Law et al., 1983). To determine the total ascorbate content 100 µL 10 mM dithiothreitol (DTT; Sigma-Aldrich) was added to 100 µL of the supernatant; after 10 min of incubation at 20 °C 100 µL 0.5% *N*-ethylmaleimid (NEM; Sigma-Aldrich) was added to remove the excess of DTT. ASC concentrations were determined by measurement of the optical density (OD) at 525 nm. Dehydroascorbate (DHA) content was calculated as a difference between the concentration of the total and the reduced ascorbate. Standard curve was obtained from reduced ASC within the 0–10 mM range.

Total glutathione (GSH) and oxidized glutathione (GSSG) concentrations were measured spectrophotometrically using an enzymatic assay (Griffith, 1980). Total glutathione content was determined directly in the tissue extract, whereas GSSG content was measured in extracts which were supplemented by 2-vinylpyridine to mask reduced GSH. GSH and GSSG concentrations were determined by measuring OD at 405 nm, using a glutathione reductase (GR) enzymatic assay (Carlberg and Mannervik, 1985). The reaction mixture contained 0.2 mM NADPH (Sigma-Aldrich), 0.25 mM 5,5′-dithiobis-2-nitrobenzoic-acid (DTNB; Sigma-Aldrich), 20 µL tissue extract and 1 U of GR (from baker's yeast, Sigma-Aldrich) in a phosphate buffer (0.1 M, pH 7.5) in a total volume of 1 mL. Reduced GSH content was calculated from the difference between the concentration of total GSH and GSSG. Standard curves were obtained for GSH and GSSG within the 0–2 µM range.

2.6. Calculation of the glutathione half-cell reduction potential

The reduction potential of the GSH/GSSG couple (half-cell reduction potential; E_{hc}) was determined with the Nernst equation using the formula of Schafer and Buettner (2001): $E_{hc} = -240 - (59.1/2)\log([GSH]^2/[GSSG])$ mV; where −240 mV is the standard reduction potential of glutathione on 25 °C, pH = 7.0.

2.7. Determination of antioxidant enzyme activities

250 mg of shoot or root tissue was homogenized on ice in 1 mL cold extraction buffer (100 mM phosphate buffer pH 7.0, containing 1 mM phenylmethylsulfonyl fluoride and 1% polyvinyl-polypyrrolidone). The homogenate was centrifuged for 20 min at 12 000*g* at 4 °C. The supernatant was used for enzyme activity assays. The protein contents of the extracts were determined by the method of Bradford (1976).

Determination of thioredoxin peroxidase (TPOX; EC 1.11.1.15) enzyme activity was based on the protocol of Horváth et al. (2015a) with some modifications. The activity was measured with cumene hydroperoxide (CHP; Sigma-Aldrich) as a substrate. The reaction mixture contained 0.2 mM NADPH, 5 µM TRXh3, 0.1 µM NADPH-dependent thioredoxin reductase (NTRa) recombinant protein produced by *E. coli* according to Marty et al. (2009), 50 µL of enzyme extract and 0.25 mM substrate in a Tris-HCl buffer (0.1 M, pH 7.4) in a total volume of 1 mL. The NADPH consumption was followed by measuring the absorbance at 340 nm. The nonspecific NADPH decrease was corrected by using additional measurements without the CHP substrate. One U was equal to nmol converted NADPH in 1 min, $\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$.

The glutathione peroxidase (GPOX; EC 1.11.1.9) activity was also measured spectrophotometrically with CHP substrate using a protocol of Horváth et al. (2015a). The reaction mixture contained 4 mM GSH, 0.2 mM NADPH, 0.05 U of GR (from baker's yeast, Sigma-Aldrich), 100 µL enzyme extract and 0.5 mM substrate in a phosphate buffer (0.1 M, pH 7.0) in a total volume of 1 mL. One U was equal to nmol converted NADPH in 1 min, $\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$.

The glutathione transferase (GST; EC 2.5.1.18) enzyme activity was measured spectrophotometrically using the artificial 1-chloro-2,4-dinitrobenzene (CDNB; Sigma-Aldrich) substrate, as published earlier (Horváth et al., 2015a). The reaction mixture contained 1 mM GSH, 100 µL enzyme extract and 1 mM substrate in a phosphate buffer (0.1 mM, pH 6.5) in a total volume of 1 mL. One U is the amount of the enzyme producing 1 nmol conjugated product in 1 min, $\varepsilon_{340} = 9.6\ mM^{-1}\ cm^{-1}$.

The guaiacol peroxidase (POX; EC 1.11.1.7) activity was determined by monitoring the increase in A_{470} during the oxidation of the guaiacol substrate (Sigma-Aldrich), according to Horváth et al. (2015b). The

reaction mixture contained 30 mM H_2O_2 (Merck Millipore), 10 µL enzyme extract and 20 mM substrate in a phosphate buffer (50 mM, pH 7.0) in a total volume of 1.5 mL. The amount of enzyme producing 1 µmol of oxidized guaiacol in 1 min was defined as 1 U, $\varepsilon_{470} = 26.6\ mM^{-1}\ cm^{-1}$.

The ascorbate peroxidase (APX; EC 1.11.1.11) activity was assayed according to the protocol of Tari et al. (2015). For the APX assay, 1 mM ascorbate (Sigma-Aldrich) was added to the extraction buffer. The H_2O_2-dependent oxidation of ascorbate was followed by a decrease in A_{290}. The reaction mixture contained 1 mM H_2O_2, 100 µL enzyme extract and 50 µM ascorbate in a potassium phosphate buffer (50 mM, pH 7.0) in a total volume of 1 mL. One U was equal to nmol oxidized ascorbate in 1 min, $\varepsilon_{290} = 2.8\ mM^{-1}\ cm^{-1}$.

The catalase (CAT; EC 1.11.1.6) activity was determined by the decomposition of H_2O_2, measured spectrophotometrically by following the decrease in A_{240} (Horváth et al., 2015b). The reaction mixture contained 100 µL enzyme extract and 20 mM H_2O_2 in a phosphate buffer (50 mM, pH 7.0) in a total volume of 1.5 mL. One U is the amount of decomposed H_2O_2 (µmol) in 1 min, $\varepsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$.

2.8. RNA extraction, expression analyses with quantitative real-time PCR

The expression rate of *Arabidopsis thaliana GPXLs* genes and selected stress related genes were determined by quantitative real-time PCR (RT-qPCR) after the purification of RNA from 100 mg plant material according to Chomczynski and Sacchi (1987), as was described in Benyó et al. (2016). The used primers are available in the Supplementary material (Table S1) of this paper. After each RT-qPCR, melting curve analysis was performed which resulted in a single product-specific melting temperature peak in every case. The *actin2* (*At3g18780*) gene exhibited constant expression in our experiments, thus it was used as internal control for data normalization (Papdi et al., 2008). Data from the RT-qPCR were calculated using the $2^{-(\Delta\Delta Ct)}$ formula (Livak and Schmittgen, 2001). To demonstrate the differences between changes in the expression levels of the investigated genes, the relative transcript level in the *Arabidopsis thaliana* WT control shoot and root samples were considered to be 0 for each gene on a $\log_2$ scale, and the results were presented on a heat map.

2.9. Correlation analysis

To determine the relationship between the different variable parameters, we calculated the Pearson's correlation coefficients. The correlation matrixes were prepared by R program (http://www.R-project.org). The values of the correlation coefficient varied between +1 and −1. When the value is around +1 or −1, it indicates a close positive or negative relationship between the variables, respectively. As the correlation coefficient value approximates 0, the relationship between the two variables will become weaker.

2.10. Statistical analysis

Data presented here are the means ± SE. All experiments were carried out at least three times. In each treatment at least three independent samples were measured, unless indicated otherwise. Statistical analysis was carried out with SigmaPlot ver. 11.0 software (Systat Software Inc., Erkrath, Germany). To compare the mean values of the WT and mutant plants, Student's t-test was used, and asterisks indicate the significant differences (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

3. Results

3.1. The effect of NaCl and isoosmotic PEG treatments on lipid peroxidation, H_2O_2 and the main non-enzymatic antioxidants levels

To compare the osmotic stress responses of the wild type and *Atgpxl* mutants, 100 mM NaCl or 200 mOsm PEG treatments were applied on 6-week-old plants for two days. The effect of the treatments is presented on Figs. S2 and S3 by choosing representative plants (Supplemental material). Rosette sizes of most mutants were slightly bigger than wild type plants under control conditions (Fig. S4). To compare the phenotype of the mutant and wild type plants under stress conditions, rosette diameters and root lengths of 6-week-old plants were measured in salt and osmotic stress conditions. Rosette and root sizes of the mutants were similar to wild type plants in both salt or osmotic stresses, with the exception of *Atgpxl6*, which had significantly smaller rosettes after salt treatment (Figs. S2–S4).

Analysis of the H_2O_2 and the lipid peroxidation marker MDA levels in 6-week-old plants grown hydroponically under control conditions revealed significantly higher H_2O_2 amounts in the shoots of *Atgpxl4, −5, −6, −7* and in the roots of *Atgpxl5* and *–8* mutants than in wild type, but there was no significant alteration in the MDA levels (Figs. 1 and 2a, d). The amounts of the reduced and total ASC were usually lower in shoots of mutant plants than in the wild type, except for *Atgpxl6* (Fig. 1g). The *Atgpxl3, −5* and *−6* mutants had elevated ASC level in their roots under control conditions, while the *Atgpxl1*, *−7* and *−8* possessed less total ASC compared to the WT (Fig. 2g). The GSH level was significantly enhanced in the roots of all mutants except for *Atgpxl2* (Fig. 2j), and in the shoots of several mutants, significantly in the *Atgpxl5, −6* and *−7* (Fig. 1j). The calculated E_{hc} value was more negative in the shoots of *Atgpxl1, −4, −5, −6, −7* and in the roots of all *Atgpxl* mutants, except for *Atgpxl2*, than in that of WT under control conditions (Figs. 1 and 2m).

Two days of 100 mM NaCl treatment did not elevate the levels of the measured oxidants neither in shoots or roots, however isoosmotic (200 mOsm) PEG concentration resulted in enhanced H_2O_2 and MDA levels in shoots and caused more dramatic damages in the plants after two days than the salt stress. The increment of oxidants was usually similar in the mutants and the wild type, but the elevation of H_2O_2 was even lower in the case of *Atgpxl2* and *–8* than in the WT shoots (Fig. 1b, c, e, f). Compared to WT, significantly higher H_2O_2 level was found only in 200 mOsm PEG-treated *Atgpxl6* roots (Fig. 2b, c, e, f), however in shoots, generally 2–3 times higher H_2O_2 and MDA levels were measured. In the PEG-treated *Atgpxl2* and *−8* shoots less H_2O_2 accumulated than in the WT (Figs. 1 and 2c, f).

In parallel, the treatments caused different changes in the ASC and GSH content. The level of ASC was generally higher in shoots than in roots, and the total ASC amount increased especially in shoots due to PEG treatment, where both the reduced ASC and DHA levels enhanced by 2–3 times in all plants. After applying PEG-induced osmotic stress, the shoots of six mutants (*Atgpxl2, −3, −4, −5, −7,* and *-8*) showed elevated level of this antioxidant, while in *Atgpxl1,* less ASC was found than in the treated wild type (Fig. 1i). In case of salt stress, the shoots of *Atgpxl2* and the roots of *Atgpxl1* and −4 were the exceptions where no significant changes occurred in the amount of total ASC (Figs. 1 and 2h). Interestingly, the ASC did not accumulate in the roots of *Arabidopsis* plants under PEG-triggered osmotic stress and almost the whole ascorbate pool was found in its oxidized (DHA) form. In addition, the *Atgpxl1*, *−3* and *−7* mutants had less total ASC in the treated roots than the wild type (Fig. 2i).

The GSH content of roots was ca. half of that in shoots of the plants, and the applied salt stress increased its level in the shoots of only three mutants (Figs. 1 and 2k, note the different vertical scales on Figs. 1 and 2). The biggest changes were detected in the roots of WT (the reduced GSH increased from 16 ± 2.2 to 81 ± 2.9 nmol g^{-1} FW, while the amount of GSSG from 1.8 ± 0.04 to 3.6 ± 0.2 nmol g^{-1} FW,

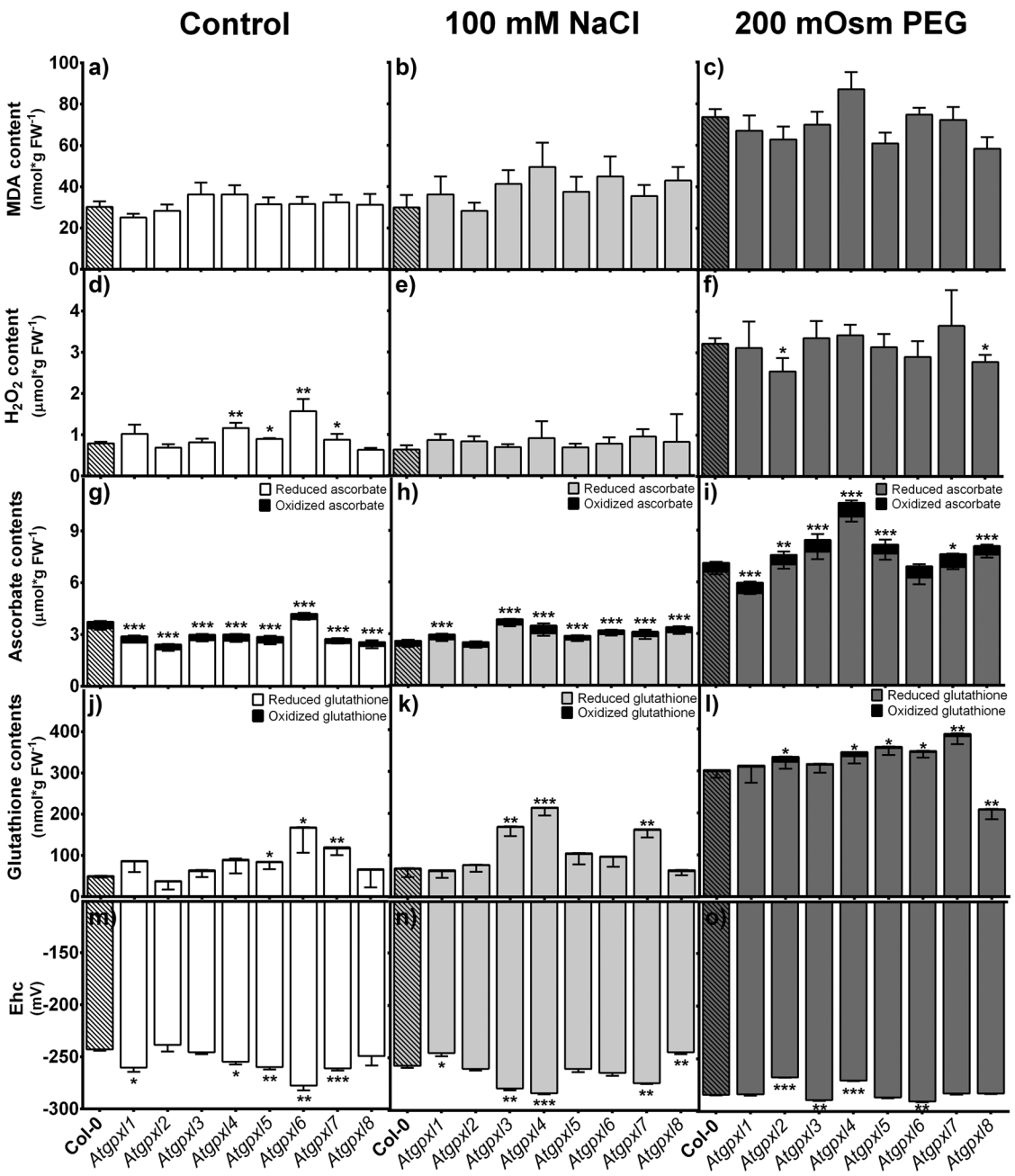

Fig. 1. Malondialdehyde (MDA), H_2O_2, ascorbate (ASC), glutathione (GSH) contents and glutathione half-cell reduction potential (E_{hc}) in the shoots of 6-week-old *Arabidopsis thaliana* wild type (Col-0) and *Atgpxl* mutants under control conditions and treated with 100 mM NaCl or 200 mOsm PEG 6000 (polyethylene glycol) for two days. In case of ASC and GSH contents the black segment of the bars represents the oxidized ascorbate (DHA) and oxidized glutathione (GSSG), respectively. The data are presented by mean values ± SE, and asterisks indicate the significant differences in each cases from wild type, determined by Student's *t*-test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

respectively). Due to the increased GSH pool in shoots or the less GSSG in the roots of some mutants, the calculated E_{hc} was more negative in several cases than that of WT (Figs. 1 and 2n), indicating that these mutants might successfully cope with the applied NaCl stress.

The PEG treatment resulted in an about 2–5 times elevation in the GSH content of the shoots (the lowest, 207 ± 13 nmol g^{-1} FW was measured in *Atgpxl8,* the highest, 382 ± 11 nmol g^{-1} FW in *Atgpxl7*). The calculated E_{hc} value was most negative in the shoots of *Atgpxl3* and *−6* (ca. −290 mV), while the E_{hc} in *Atgpxl2* and *−4* shoots were with ca. 20 mV more positive (−267 ± 0.4 and −270 ± 0.6 mV, respectively) than that of the WT (Fig. 1l, o). In roots, 2 days after applying the PEG the GSH level in WT and *Atgpxl4* roots was similar to that in untreated controls, but in other cases it was decreased. The calculated E_{hc} value was the most negative in the roots of *Atgpxl4* (−239 ± 2.3 mV), although in the roots of *Atgpxl1, −2, −3* and *−7* the redox state of mutants became more oxidized under osmotic stress (Fig. 2l, o), indicating that they tolerate the PEG-triggered stress less than the wild type plant.

3.2. Changes in the antioxidant enzyme activities

To investigate the enzymatic antioxidants and their role in the maintained ROS level and redox homeostasis in the *Atgpxl* mutants, some important enzyme activities were measured.

Determination of the GPOX activity using GSH as a reducing agent and the lipid peroxide model substrate (CHP) resulted in 17–22 nmol NADPH mg^{-1} protein specific activity in shoots, while this value was between 47 and 73 U mg^{-1} protein in roots, which could be increased

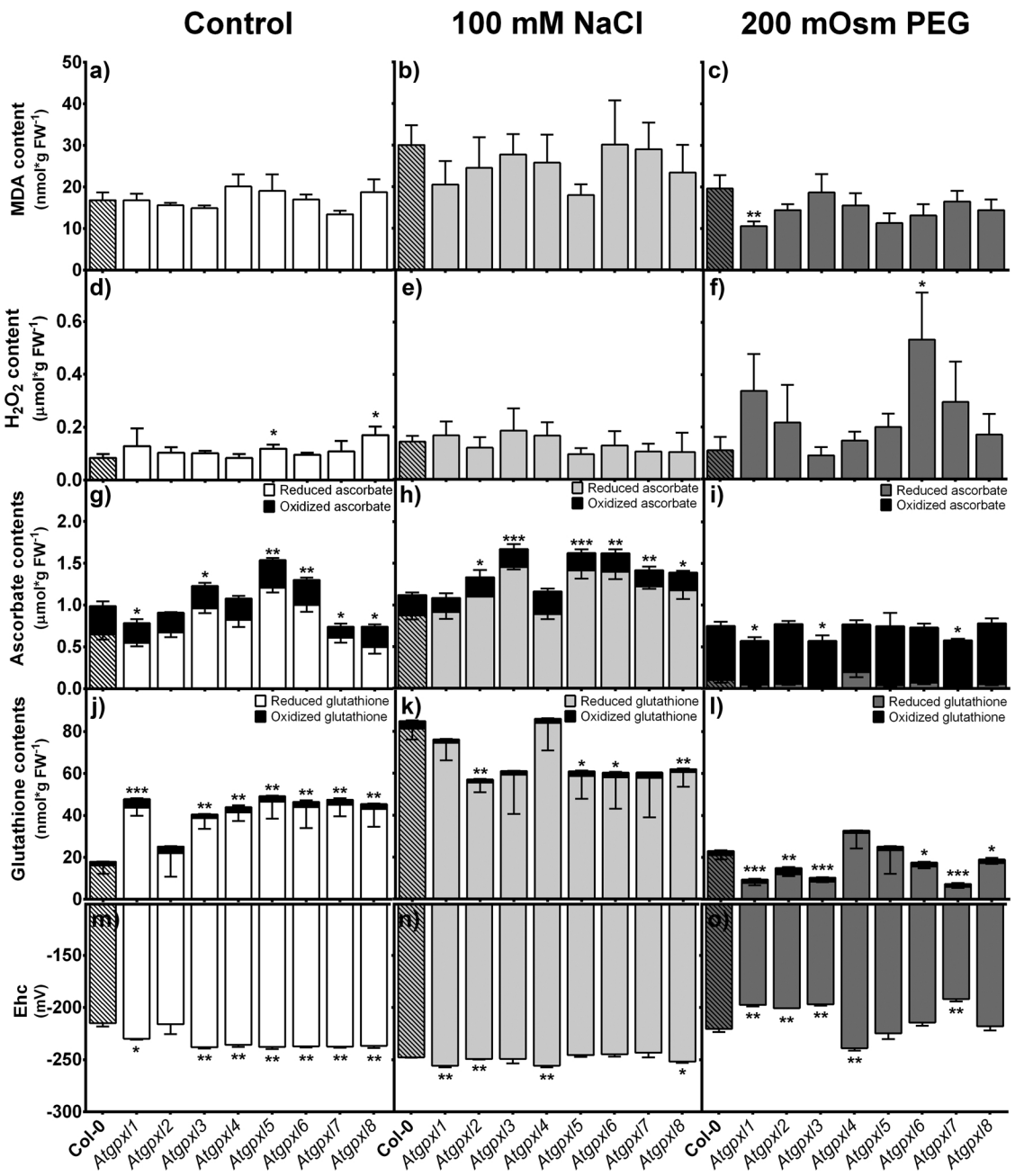

Fig. 2. Malondialdehyde (MDA), H_2O_2, ascorbate (ASC), glutathione (GSH) contents and glutathione half-cell reduction potential (E_{hc}) in the roots of 6-week-old *Arabidopsis thaliana* wild type (Col-0) and *Atgpxl* mutants under control conditions and treated with 100 mM NaCl or 200 mOsm PEG 6000 (polyethylene glycol) for two days. In case of ASC and GSH contents the black segment of the bars represents the oxidized ascorbate (DHA) and oxidized glutathione (GSSG), respectively. The data are presented by mean values ± SE, and asterisks indicate the significant differences in each cases from WT, determined by Student's *t*-test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

up to 133 U mg^{-1} protein due to PEG treatment (Figs. 3 and 4d–f). There were no significant differences between the GPOX activity in shoots of the wild type and *Atgpxl* plants, moreover in the *Atgpxl6* and *−7* roots the GPOX activity was even higher than in the untreated wild type. Many of the GST enzymes also have GPOX activity, but measuring the GST activity with the artificial CDNB substrate showed similar pattern to the GPOX activity without significant changes in mutants under control conditions (Figs. 3 and 4g). However, detecting the TPOX activity with thioredoxin h3 as a reducing compound and CHP substrate revealed that in most cases the specific TPOX activity is decreased in the shoots of mutant plants (exceptions are *Atgpxl5* and *−7*), verifying that the thioredoxin peroxidase activity of the AtGPXL enzymes is their important feature. The lowest value was measured in *Atgpxl8*, which exhibited about half of the TPOX activity shown in WT shoot. There were similar TPOX activities in shoots and roots (in the wild type the specific activities were 9.5 ± 3.0 and 10.9 ± 2.9, respectively), but in roots only the *Atgpxl2* had lower TPOX activity than the WT under control conditions (Figs. 3 and 4a). Applying salt or osmotic stresses caused relative small changes and rather similar patterns of the activities of the investigated antioxidant enzymes. It revealed that, in contrary to the WT, the TPOX enzyme activity did not increase in several mutants (e.g. in roots after NaCl treatment, the only exception was *Atgpxl7,* or after PEG treatment in shoots of *Atgpxl1*, *−4* and *−8*), however, in some cases even higher TPOX activity was measured in the GPXL mutants (Figs. 3 and 4). Some examples indicated that the lower GPOX activity is compensated with elevated GST activity (e.g. at the PEG-treated roots, Fig. 4f, i). Among the H_2O_2-related enzymes the APX activity differed substantially in the mutant plants: while its activity

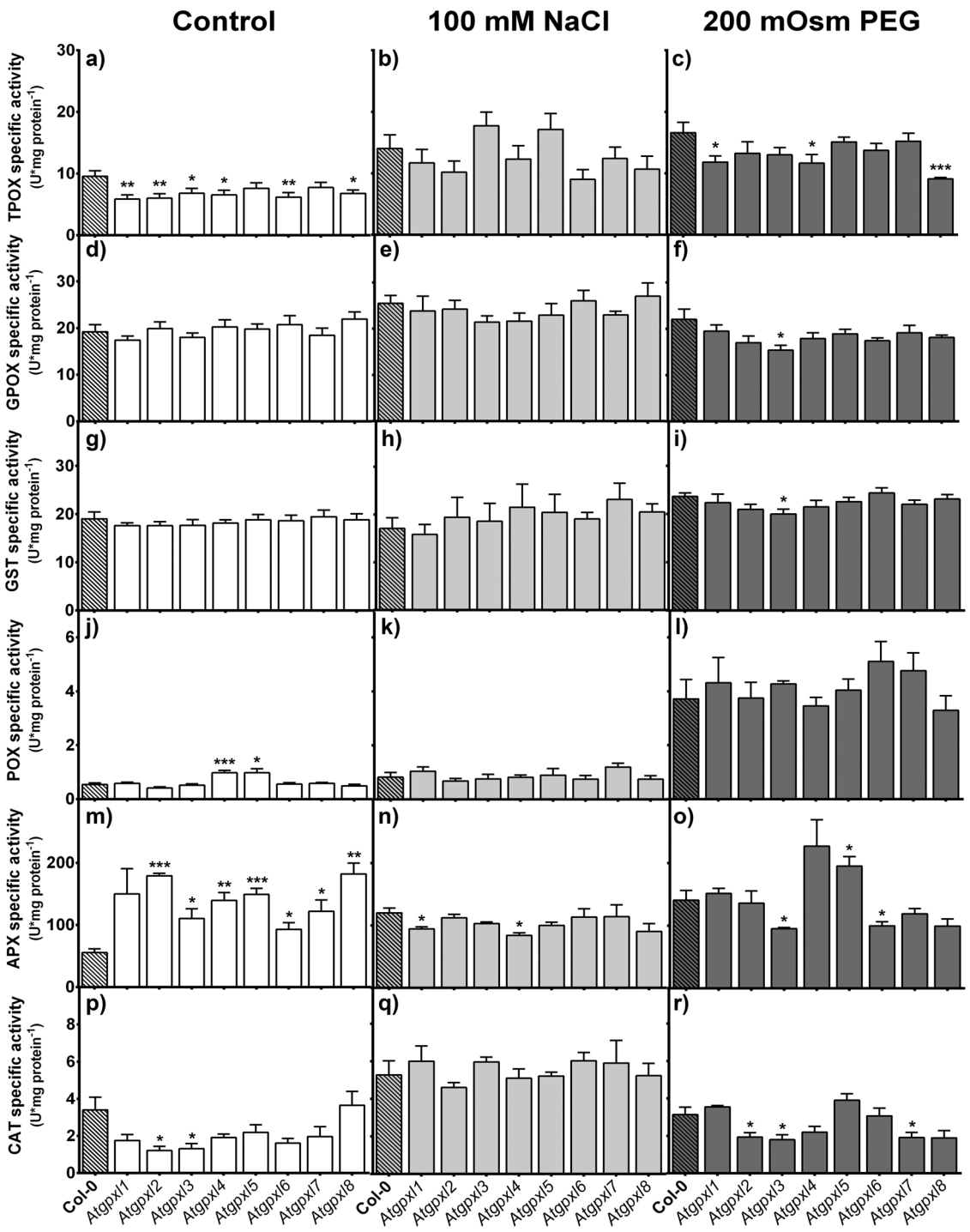

Fig. 3. Antioxidant enzyme activities in the shoots of 6-week-old *Arabidopsis thaliana* wild type (Col-0) and *Atgpxl* mutants under control conditions and treated with 100 mM NaCl or 200 mOsm PEG 6000 (polyethylene glycol) for two days. Thioredoxin peroxidase (TPOX) and glutathione peroxidase (GPOX) specific activities were measured with cumene hydroperoxide (CHP) substrate, glutathione transferase (GST) specific activity with 1-chloro-2,4-dinitrobenzene (CDNB), guaiacol peroxidase (POX) specific activity with guaiacol, ascorbate peroxidase (APX) specific activity with ascorbate and catalase (CAT) specific activity with H_2O_2 substrates. The data are presented by mean values ± SE, and asterisks indicate the significant differences in each cases from wild type, determined by Student's *t*-test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

increased in the shoots of mutants, it was generally lower in their roots than that in the WT but, parallelly with the increased DHA contents, it was induced in most of the mutant roots due to stress treatments (Figs. 3 and 4).

3.3. Expression patterns of AtGPXL and some stress-related transcription factor genes

Hereafter we estimated the possible redundancy detectable among the AtGPXLs. The sites of insertion in the investigated mutants and the constructed forward and reverse oligonucleotides used for RT-qPCR experiments are presented in Table S1 (Supplemental material). Analysis of the transcript level of *AtGPXLs* in the hydroponically grown 6-week-old *Arabidopsis* plants revealed that the *AtGPXL* genes expressed in leaves at higher levels than in roots (Table S2, Supplemental material). The order of AtGPXL transcript amounts in the wild type plants were *AtGPXL1, −2, −8, −7, −6, −5, −3, −4* in shoots and *AtGPXL2, −8, −6, −1, −5, −3, −7* and *−4* in roots. In wild type plants salt

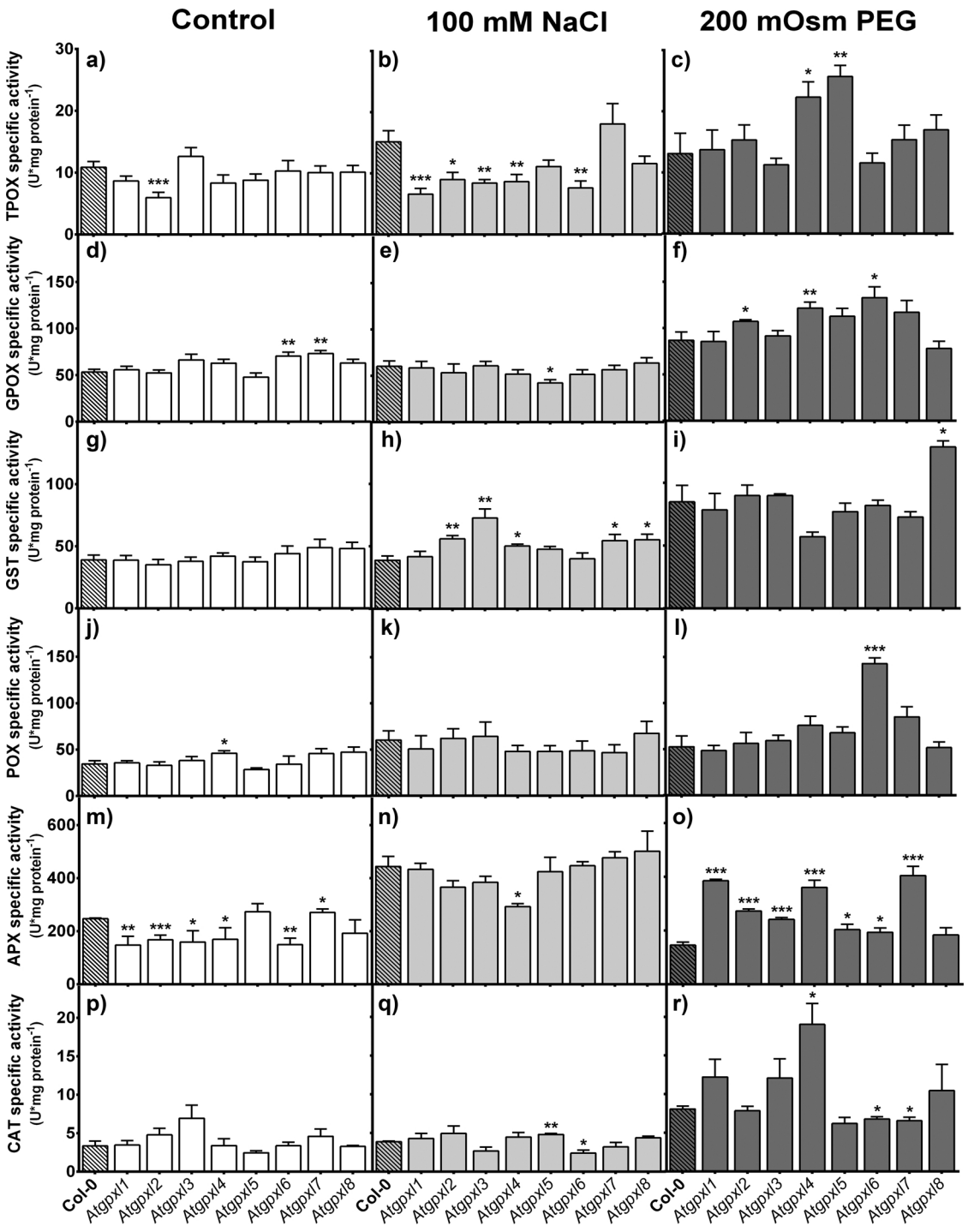

Fig. 4. Antioxidant enzyme activities in the roots of 6-week-old *Arabidopsis thaliana* wild type (Col-0) and *Atgpxl* mutants under control conditions and treated with 100 mM NaCl or 200 mOsm PEG 6000 (polyethylene glycol) for two days. Thioredoxin peroxidase (TPOX) and glutathione peroxidase (GPOX) specific activities were measured with cumene hydroperoxide (CHP) substrate, glutathione transferase (GST) specific activity with 1-chloro-2,4-dinitrobenzene (CDNB), guaiacol peroxidase (POX) specific activity with guaiacol, ascorbate peroxidase (APX) specific activity with ascorbate and catalase (CAT) specific activity with H_2O_2 substrates. The data are presented by mean values ± SE, and asterisks indicate the significant differences in each cases from wild type, determined by Student's *t*-test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

treatment enhanced the expression of *AtGPXL2*, *AtGPXL6* and *AtGPXL8* and reduced *AtGPXL1* and *AtGPXL4* transcripts in the shoots. In salt-treated roots *AtGPXL1*, *AtGPXL3* and *AtGPXL8* transcript levels were higher. PEG led to enhanced expression of *AtGPXL4* in shoots and roots, while *AtGPXL5* expression was reduced in roots (Fig. 5). The expression pattern of *GPXL* genes changed notably in the mutants (Fig. 5, Table S2). The only exception is the *Atgpxl1*, which has the insertion in its 3′ regulator region (Fig. S1; Polymorphism: SALK_128885.41.65.n), thus the gene product may have relatively small defects. Interestingly, we have found only a few examples for induced expression of other *GPXLs* in the mutants among the untreated plants, rather the expression of more than one *AtGPXL* gene was down-regulated in several mutants compared to WT. Joint down-regulation of at least four *AtGPXLs* was detected in the shoots of *Atgpxl4* and *−8*, and in roots of *Atgpxl5* under control conditions (Fig. 5). For example, in the *Atgpxl8* shoots the transcript levels of *AtGPXL1*, *-4, −5* and *−7* decreased along with that of the mutant gene.

Two days of 100 mM NaCl treatment in the shoots of the wild type

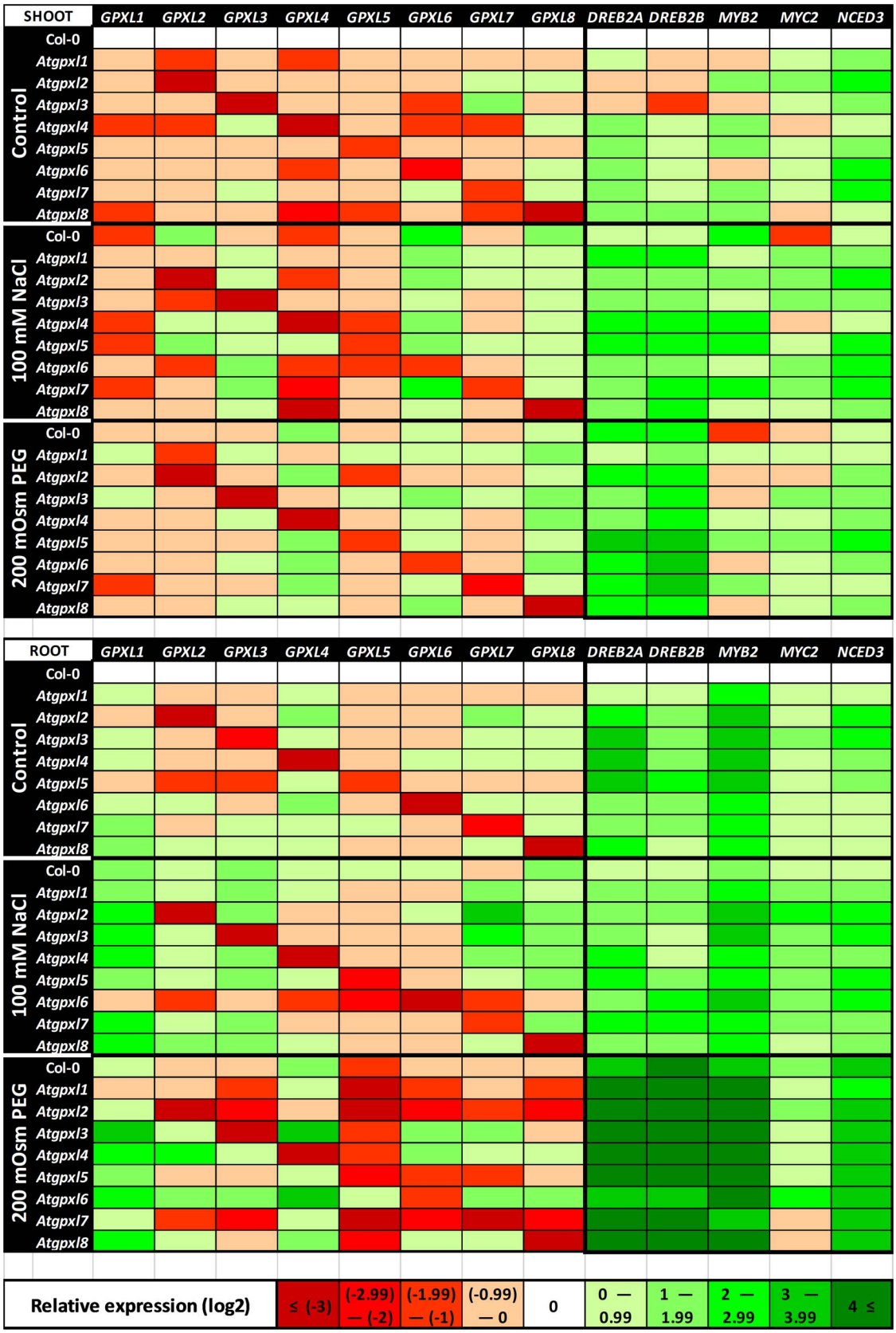

Fig. 5. Relative expression of *AtGPXL1-8* genes (*At2g25080, At2g31570, At2g43350, At2g48150, At3g63080, At4g11600, At4g31870, At1g63460*, respectively) and stress related genes [*DREB2A (At5g05410) DREB2 B (At3g11020), MYB2 (At2g47190), MYC2 (AT1G32640), NCED3 (AT3G14440*) in the shoot and root of 6-week-old *Arabidopsis thaliana* wild type (Col-0) and *Atgpxl* mutants under control conditions and treated with 100 mM NaCl or 200 mOsm PEG 6000 (polyethylene glycol) for two days. The *glyceraldehyde-3-phosphate dehydrogenase C2* (*GAPDH2; At1g13440*) and *actin2* (*At3g18780*) genes were used as internal controls, data were normalized to the control wild type values (0) and presented on a $\log_2$-scale as a heatmap. Red colours show repression, while green colours show activation, according to the colour scalebar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

induced the expression levels of *AtGPXL6* and, to a lesser extent, that of the *AtGPXL2* and *AtGPXL8*. In the roots the transcript amounts of *AtGPXL1*, *AtGPXL3* and *AtGPXL8* were increased, but the expression of *AtGPXL1* and *AtGPXL4* was down-regulated (Figs. 3 and 4). The iso-osmotic PEG treatment induced only the *AtGPXL4* both in the shoots and roots of WT, and down-regulated the *AtGPXL5* expression in roots. Co-regulation of several *ATGPXL* genes was indicated, e. g. both in the shoot and root of the *Atgpxl6* mutant after applying salt stress, but after the PEG treatment in the *Atgpxl1*, *−2* and *−7* roots simultaneous decrease of the *AtGPXL*s expression levels was detected (Fig. 5).

Comparing the expression patterns of *GPXL* genes two days after applying the salt stress highlighted that in shoots the transcript amounts of *AtGPXL6* increased in 6 genotypes, meanwhile that of other *AtGPXLs* (the most frequently *AtGPXL4*) decreased. However, in roots the expression of *GPXL1, −3, −7* and *−8* were more often induced, indicating their role in salt stress responses (Figs. 3 and 4). On the contrary, after the isoosmotic PEG treatment the transcription of the *AtGPXL4* and *−8* was up-regulated generally in the shoots, while the *AtGPXL1* and *−4* in the roots. Another important differences between the osmotic stress responses of the shoots and roots was the down-regulation of the *AtGPXL5* expression in most of the treated roots (Fig. 5).

Nevertheless, the joint up- and downregulation of several *AtGPXL* genes implicated the possibility of a common regulation and being part of signaling pathways. The expression patterns of some salt-, dehydration or ABA-induced transcription factor (TF) coding genes, such as *DREB2A, DREB2B*, *MYB2*, *MYC2,* and one gene coding a key enzyme in ABA biosynthesis (nine-*cis*-epoxycarotenoid dioxygenase3, *NCED3,*) were also determined by RT-qPCR in the control and treated wild type and insertional mutant plants (Fig. 5). The transcript amounts of the investigated TFs were enhanced in most of the *Atgpxl* mutants even under control conditions both in shoots and roots, although significantly elevated *AtMYC2* expression was found only in *Atgpxl2* shoots and *Atgpxl3* roots (the transcript levels increased by 2 or 4 times, respectively). Moreover, the applied NaCl or PEG treatments caused much higher induction of *DREB2A*, *DREB2B* and *NCED3* in most of the mutant's shoots, while in roots in addition the *MYB2* was usually strongly upregulated (Fig. 5).

These results also revealed organ specific differences, thus we wondered how the measured parameters correlate in shoots and roots. Correlation analysis among all physiological parameters measured in the investigated plants and treatments (including H_2O_2 and MDA levels, oxidized and reduced ascorbate and glutathione, the activities of the antioxidant enzymes and the calculated E_{hc} values under control, salt stressed and osmotic stressed conditions) in the wild type shoots and roots are demonstrated on Fig. 6. Highlighting the GPOX activity in the wild type shoots, this parameter showed close or medium positive correlation with CAT, APX and TPOX activities ($r = 0.84$, 0.68 and 0.58, respectively) and weak negative with E_{GSH} ($r = -0.29$), while in roots very close positive correlations were found between GPOX and DHA levels, CAT and GST activities ($r > 0.9$), and weak positive with TPOX and E_{GSH} ($r \approx 0.2$). However, there was a very close negative correlation between the TPOX activity and E_{GSH} both in the shoot and root of the WT. The TPOX activity correlated positively with most of the measured physiological parameters in shoot (except the CAT activity), but in root there was a different pattern with less positive correlations (Fig. 6). The correlation analysis based on the ASC, GSH levels, GPOX, TPOX activities and, additionally, considering the investigated gene expression levels in shoots and roots of all *Atgpxl* genotypes is presented in Table S2 (Supplemental material).

4. Discussion

GPXs are the major antioxidant enzymes in animals, responsible for protecting the cells against oxidative stress via reduction of H_2O_2 and organic hydroperoxides using reduced glutathione (Maiorino et al., 1995) or thioredoxin as substrate (Björnstedt et al., 1994). In plants, H_2O_2 elimination is mainly carried out by APX and CAT isoenzymes (Ozyigit et al., 2016). The importance of POXs (Class III peroxidases) is thought to be mainly the oxidation of various substrates in the presence of H_2O_2, moreover they also may produce ROS (Passardi et al., 2005; Cosio and Dunand, 2008; Csiszár et al., 2012). Concerning the plant glutathione peroxidases, heterogeneous information can be found in the literature about their roles and significance. They contain Cys in their catalytic site instead of selenocysteine in mammalian enzymes (Eshdat et al., 1997) and it was suggested that they are more efficient in reducing peroxides other than H_2O_2, such as lipid peroxides (Milla et al., 2003). The catalytic efficiencies of tomato and sunflower GPXs proved to be higher in the presence of thioredoxin (K_M is 2.2 and 1.5 μM, $V_{max} = 263.2$ and 243.9 nmol $mmin^{-1}$ mg^{-1}, respectively) than in the presence of reduced glutathione ($K_M = 9300$ and 4900 μM, $V_{max} = 48.8$ and 46.7 nmol min^{-1} mg^{-1}, respectively) (Herbette et al., 2002). Other results even indicated that plant GPXs use only TRX, but not GSH in the reduction of hydroperoxides (Jung et al., 2002; Iqbal et al., 2006; Herbette et al., 2007). In our experiments the measured specific GPOX activities of the hydroponically grown 6-week-old wild type *Arabidopsis* shoots and roots were two- and five-fold higher, respectively than the specific TPOX activity using the same CHP substrate (Figs. 2 and 3). Mutation of one of the eight *AtGPXLs* resulted in significantly decreased TPOX activity in untreated shoots in most of the investigated T-DNA insertion genotypes, but the GPOX activity did not change or was even higher in the roots of the mutants than in the wild type. The phenotypes were more or less similar to those of the wild type in all mutant lines under the used short-day conditions, suggesting that these isoenzymes are at least partially redundant or other components of the antioxidant system can compensate the loss of a particular GPXL (Figs. 2, 3, S2, S3).

Passaia et al. (2014b) reported that there are some differences in the number of rosette leaves and lateral roots of the 4-week-old *Atgpxl* plants and they demonstrated discrete roles of individual isoenzymes in the development. As an example, the involvement of AtGPXL7 was indicated both in shoot development and in the hormone-mediated (auxin, strigolactone, ABA) control of lateral root development (Passaia et al., 2014b). Moreover, the rice *Osgpxl3* knock down mutant plants displayed short root and shoot phenotypes, and increased H_2O_2 production in root tissues compared to the wild type plants (Passaia et al., 2013) The *Osgpxl5* mutants had lower germination rate, reduced growth, and less filled grains compared to the wild-type plants (Wang et al., 2017). The mitochondrial OsGPXL1 is essential for both normal *Oryza sativa* shoot development and seed production (Passaia et al., 2014a), and root development and photosynthesis (Lima-Melo et al., 2016). These results indicate that in rice the OsGPXLs are important in H_2O_2 elimination and also influence the signaling.

Comparing the effect of osmotic stress with salt stress on the *Atgpxl* T-DNA insertion mutants revealed that the antioxidant responses of the plants have significantly changed. Interestingly, there were only smaller alterations among the plants concerning most of the measured physiological parameters, including H_2O_2 and MDA amounts and GPOX activity, either under control conditions or after 2 days of stress treatments (Figs. 1–4). While APX activity was elevated in several mutants, activities of GST, GPOX and thioredoxin peroxidases could at least partially compensate the functions of damaged GPXLs, important changes were detected in the levels of non-enzymatic antioxidants. While in the shoots the amount of the reduced ASC varied generally, in the roots GSH levels were more variable.

ASC and GSH are the major constituents of the antioxidant defense systems. Their principal function is to control the ROS accumulation through a network of reactions in the Foyer–Halliwell–Asada cycle (Foyer and Noctor, 2011; Foyer and Noctor, 2013). However, they can be regarded as key compounds that dynamically work at the cross-point between stress perception and physiological responses (Paciolla et al., 2016). Because the antioxidants determine the extent and the

specificity of ROS signals due to antioxidant redox buffering, they ultimately regulate the redox-dependent signaling pathways, deciding cell fate (De Pinto et al., 2006). However, a tight control is necessary to balance these processes, thus also antioxidant systems are finely regulated to permit variations in ROS levels in order to make appropriate signaling functions easy (Munné-Bosch et al., 2013).

According to our results, two days after applying 200 mOsm PEG treatment neither the ASC nor the GSH content increased in the roots of 6 weeks old plants (Fig. 2i, l). Because roots are the site where the applied stresses occurred first, this can be an important difference between the salt- and PEG-induced stress responses. During the investigation of the hardening mechanism of salicylic acid pre-treatments that trigger salt stress tolerance of tomato plants earlier we have observed that in the roots the APX activity was low and the ROS were non-enzymatically scavenged by ascorbate. The proper SA concentration, which led to increased salt tolerance, maintained the redox status of ASC under high salinity, furthermore enhanced GR activities and thus the reduced GSH pool in roots (Tari et al., 2015).

It was suggested earlier that ascorbate is the redox molecule which primarily regulates development, and glutathione is involved in plant development, but is mainly important for stress defense and signaling (Potters et al., 2002; Foyer and Noctor, 2005; Potters et al., 2010). Furthermore, GSH is a crucial regulator of the cell cycle and cell differentiation (Bashandy et al., 2010; Diaz Vivancos et al., 2010), and increased GSH levels are necessary for the G1-S-phase transition of the cell cycle, but enhanced GSSG levels lead to arrested cell proliferation. The absolute glutathione concentration and the ratio of [GSH]:[GSSG], that determine the glutathione redox potential, are important parameters which can be regarded as indicators of the endogenous state of plants (Meyer and Hell, 2005). The glutathione redox potential has been suggested to act as a key determinant of cell death and dormancy in plants (Kranner et al., 2006). Our correlation analysis performed among the physiological parameters measured in control and osmotic stress-treated *Arabidopsis* plants revealed strong positive correlation between both the ASC and GSH levels and the measured H_2O_2 and MDA amounts in shoots of all genotypes, but in case of roots only the correlation between the GSH and the peroxide levels was strong positive (Fig. 6, Table S2, Supplemental material). The correlation analysis based on GSH levels after different treatments showed strong positive correlations with the activity of APX and POX, but not with that of CAT and GPOX. Interestingly, between GSH and TPOX also positive correlations were found in the shoot and root of the wild type plants, but in the roots of the *Atgpxl* mutants the TPOX activity increased if the GSH content decreased (Table S2, Supplemental material).

Plants contain a large number of thioredoxins, which are some of the central players of thiol-disulfide homeostasis and have important roles in plant growth, development and chloroplast development (Gelhaye et al., 2005; Benitez-Alfonso et al., 2009). Loss of function *thioredoxin h9* mutant plants are dwarf with small yellowish leaves (Meng et al., 2010). There is increasing evidence for interactions between the glutathione and thioredoxin systems to determine the cellular redox homeostasis (Marty et al., 2009; Lu and Holmgren, 2014). Bashandy et al. (2010) found that these two thiol reduction pathways interfere with developmental processes through modulation of auxin signaling. Diaz Vivancos et al. (2010) reported that *Arabidopsis root meristemless1 (rml1)* mutants with severe GSH deficiency were unable to maintain the root apical meristem, however the shoot apical meristem was less affected, probably because of thioredoxin dependent control (Diaz Vivancos et al., 2010). Low glutathione availability affected not only the expression of PIN and auxin response genes but the antioxidant enzymes too. Schnaubelt et al. (2015) proved that in *rml1* mutant the expression of several GSH- and TRX-related genes, among them *GSTs*, *GPXLs* and glutaredoxins changed. Regarding *GPXLs*, the *AtGPXL1* and *AtGPXL7* were downregulated, while *AtGPXL6* was upregulated in *rml1* shoots, compared to wild type plants (Schnaubelt et al., 2015). Comparing the expression patterns of *AtGPXL* genes in WT with the insertion mutants two days after applying salt stress we found that in shoots the transcript amounts of *AtGPXL6* increased in 6 genotypes, meanwhile the expression of other *AtGPXLs* (the most frequently *AtGPXL4*) decreased. However in roots the *GPXL1, −3, −7* and *−8* were more often upregulated, indicating their role in salt stress responses (Figs. 3 and 4). Contrarily, after the isoosmotic PEG treatment the expression of the *AtGPXL4* and *−8* induced generally in shoots, while that of the *AtGPXL1* and *−4* in roots. Another important observation might be the root specific down-regulation of *AtGPXL5* expression after applying NaCl or osmotic stress (Figs. 3 and 4). The *Atgpxl1* mutant used in our experiments contain the T-DNA insertion in the 3′-UTR region (Fig. S1). T-DNA insertion reduced transcript levels of the *AtGPXL1* gene to half in non-treated plants, suggesting that it can be considered as a knock-down mutation, leading to a mild phenotype.

It was indicated that salt and osmotic stress responses are linked to signaling crossroads to modify root growth and development, thus avoiding salted area or maintaining ion homeostasis (Galvan-Ampudia and Testerink, 2011). In this process the role of altered hormone (e.g. auxin, cytokinins, strigolactone, ethylene and ABA) synthesis, transport or sensitivity were suggested (Fukaki and Tasaka, 2009; Takahashi et al., 2009; Galvan-Ampudia and Testerink, 2011). By *in silico* analysis earlier we have found several stress- and hormone-related *cis*-acting regulator elements of the promoter regions of *AtGPXL* genes (Bela et al., 2015). Among them the MYB binding site was found in the 5′ regulatory regions of *AtGPXL2, −3, −5, −6, −7* and *−8*, the stress related TC-rich repeats in the promoters of *AtGPXL1, −2, −6, −7* and *−8* genes, auxin and MeJA response elements were found in *AtGPXL2, −6, −7* and *−8*, ethylene responsive element (ERE) was present in AtGPX1, −4 and −6, ABA responsive elements (ABRE, CE1) were found in the upstream regulatory region of *AtGPXL1, −2* and *−6* (Bela et al., 2015).

To investigate how the expression of *AtGPXLs* could be related to the signaling events in the mutants, the transcription level of some abiotic stress-related TF genes was also determined. The DREB (Dehydration-responsive element-binding) proteins specifically interact with *cis*-acting dehydration-responsive element/C-repeat (DRE/CRT), and are involved in cold and drought stress-responsive gene expression in *A. thaliana* (Sakuma et al., 2006). It was reported that both *DREB2A* and *DREB2B* TF genes strongly induced in roots by high-salt stress and in stems and roots by dehydration stress (Nakashima et al., 2000). AtMYB2 (MYB domain protein 2) controls the ABA induction of salt and dehydration responsive genes (Abe et al., 2003; Dubos et al., 2010), while AtMYC2 (MYC domain protein 2) is a common transcription factor of light, ABA, and JA signaling pathways in Arabidopsis (Yadav et al., 2005), but both genes are induced by drought and by ABA treatment (Abe et al., 2003).

Although in our experiments the *DREB2* genes were up-regulated after 2 days of salt- and PEG-induced osmotic stress treatments both in shoots and roots of the wild-type plants, the *MYB* expression was reduced after PEG treatment and the *MYC2* was also down-regulated in the shoot after both treatments. However, the expression of the examined transcription factors was altered in the mutant plants even under control conditions: in roots the genes were generally activated, but in shoots in most cases we experienced down-regulation of 1–3 genes among these abiotic stress-related TFs (exceptions are *Atgpxl5* and *−7*) (Fig. 5).

Passaia and Margis-Pinheiro (2015) suggested that the plant GPXLs may be involved in the redox signaling and even act as redox sensor proteins. Redox signal transduction is a complex feature of aerobic life, the multiplicity of which enriched through evolution in order to balance the aftermath of metabolism and the environment. GPX(L)s can be a key player of this complex system because they are capable of sensing ROS- or redox state (Milla et al., 2003; Miao et al., 2006; Passaia and Margis-Pinheiro, 2015), interacting with other proteins and several studies supported their signaling functions (Delaunay et al., 2002; Miao et al., 2006). For example, Miao et al. (2006) reported that the AtGPXL3 interacts with protein phosphatase type 2C (PP2C) proteins in order to

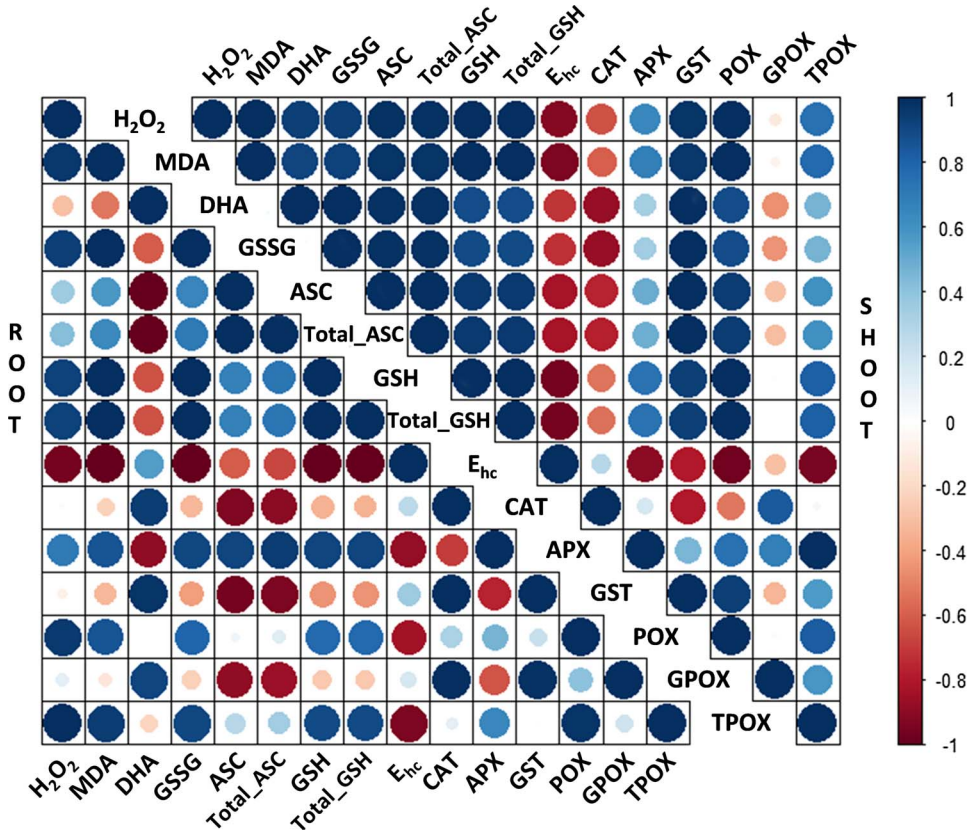

Fig. 6. Correlation analysis among the measured physiological parameters under control conditions and 2 days after applying 100 mM NaCl or 200 mOsm polyethylene glycol (PEG 6000) treatments in the shoot and root of hydroponically grown 6-week-old *Arabidopsis thaliana* wild type (Col-0) plants. Blue colours show positive, red colours show negative correlation, according to the colour scalebar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

activate plasma membrane Ca^{2+} and K^+ channels that facilitate ABA dependent stomatal closure. Mutations in *ATGPXL3* impaired the ROS homeostasis and the ABA signaling cascade, thus the function of AtGPXL3 as both a ROS scavenger and a redox transducer was indicated (Miao et al., 2006; Miao et al., 2007). Overexpression of two putative wheat *GPXL* genes in *Arabidopsis* led to altered expression levels of salt stress- and ABA related regulator genes, thereby indicating the role of GPXLs in salt- and ABA signaling (Zhai et al., 2013).

Our results also demonstrate altered signaling processes both in control conditions and after applying salt and osmotic stress. The expression patterns of both the *AtGPXLs* and the selected stress-related TF genes in the investigated *Atgpxl1-8* mutants differed from that of the wild type both under control and stress conditions (Fig. 5). Interestingly, several *AtGPXLs* were down-regulated in the *Atgpxl1-8* mutants, but the *DREB2A*, *DREB2B*, *MYB2* genes, and the NCED gene (which is involved in ABA biosynthesis) were usually upregulated. The down-regulation of the *AtGPXLs* and the up-regulation of the stress-inducible TF genes was observable especially in the roots of PEG-treated mutants, excluding *Atgpxl1* (Fig. 5). However, their interaction and the nature of their relationship requires further investigations. Although the correlations among GPOX and TPOX activities and other stress-related parameters measured under different conditions varied in the wild type and the *Atgpxl* mutants, our results indicate the strong connections with the ASC and GSH systems and thus with the redox state (Table S2).

5. Conclusion

Our results concerning the ROS elimination and antioxidant responses of *Atgpxls* revealed the importance of AtGPXLs in determining the redox homeostasis of cells beside their role played in the elimination of peroxides. While changes in ASC and GSH pools and in the expression of some abiotic stress-related TF genes allude to their relevance even under normal conditions, applying salt- and osmotic stress treatments on wild type and T-DNA insertion mutant *A. thaliana* plants grown in Hoagland solution proved to be a suitable system for verifying their involvement in determining both the total GPOX and the TPOX activities. Mutation in a single *AtGPXL1-8* gene did not cause drastic changes in the stress-related physiological parameters even after 2-day-long 100 mM NaCl or 200 mOsm PEG-induced osmotic stress treatments. Although there were some indications that the activities of other antioxidant enzymes, such as APXs, GSTs and TRXs, may compensate the damaged function of GPXLs in the mutants, insertion of the T-DNA into AtGPXL1-8 genes resulted in a decrease rather in the TPOX than in the GPOX activity. The changes exhibited organ-specificities; for example more *AtGPXLs* were down-regulated in the shoots of untreated mutants than in their roots. The connection between the AtGPXLs and the GSH pool seemed to be stronger in roots than is shoots, and the relevance of GPOX activity was generally higher in the roots than in shoots. Our results also indicate that the AtGPXLs may function both as a ROS scavenger and as a redox transducer and can be the link between the GSH and TRX redox systems.

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Appendix A. Supplementary data

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